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13. ABSTRACT *(Maximum 200 Words)*

Telomeres are repetitive sequences that protect the ends of linear chromosomes and shorten during each cell division. Very short telomeres have been associated with changes in gene expression (in yeast) and decreased genomic stability. In the first year we published the first proof that silencing effects can occur at human telomeres. A luciferase reporter near a telomere showed on average a 10-fold reduction in expression relative to internal control genes. Furthermore, we showed that the silencing is reversible through inhibition of histone deacetylases and that the strength of silencing is dependent on telomere length. Only 5-10% of breast cancers are hereditary and very little is known about the factors influencing sporadic cases. Further study of gene expression near telomeres will help determine whether telomere length could play a role in the progression of breast cancer.

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Introduction

Telomeres are repetitive sequences that protect the ends of linear chromosomes. Telomeres shorten with each cell division, eventually reaching a critically short length that causes the cell to permanently cease dividing after reaching number of divisions known as the Hayflick limit. It has been shown in our lab and others that this telomere initiated growth arrest (termed M1) is mediated through the tumor suppressors p53 and pRb. Inhibiting these proteins with SV40 T antigen or HPV E6/E7 leads to further division until a second limit (M2) is reached. This proliferation in the presence of short telomeres is associated with end to end chromosome fusions and genomic instability (1,2). This genomic instability occasionally results in the production of a rare clone that can continue to divide due to the activation of telomerase, an enzyme capable of adding new DNA to the telomeres. This situation may parallel the formation of cancerous cells *in vivo*, which in many cases show evidence of genomic rearrangements near telomeres and sometimes centromeres, losses of entire chromosomes, and activation of telomerase (1-4). Dicentric chromosomes have been shown to have little or no telomeric DNA present at the junction in humans (5) and mice (2). Note that telomere shortening is correlated with aging, and aging is one of the most important risk factors for sporadic breast cancer. Evidence for telomere length as an important regulator of the intracellular environment beyond its role as a "mitotic clock" originally came from yeast. In Saccharomyces cerevisiae, it has been shown that a transgene integrated near a telomere will be transcriptionally silenced by an active mechanism involving SIR proteins that depends both on the length of the telomere and on the distance to the transgene (6). More recently, an endogenous gene has been characterized that exhibits this silencing, or telomere position effect (TPE), in yeast (7). Our work during this first year of this grant has extended these observations from yeast to human cancer cells.

Body

"Task 1" from my statement of work was to establish the existence of telomere position effects in human cancer cells through studies on the behavior of a luciferase reporter gene inserted either at a telomere or at an internal (control) site. In the months preceding the start of funding and during the first year, I completed plasmid-based vectors for inserting a luciferase reporter gene either at the telomere or at an internal site. During the process, over 100 clones were screened for insertions sites (by PCR) and transgene expression and a subset of these were selected for further analysis. The telomeric or internal location of the transgene was then verified by Southern blot, which also showed the length of the adjacent telomere for telomeric insertion sites. Expression was compared between the two groups and it was found that in spite of a large degree of variation within each group (as expected with stable transfectants), telomeric clones expressed the telomeric reporter at a ten-fold lower level on average as compared to internal controls. Partial repression by tetracycline was not found to affect either telomeric or internal clones preferentially and was not pursued further. The rate of induction or repression of luciferase expression using the tetracycline-inducible (tet-off) system was not affected by the position of the transgene. The effect of adjacent telomere repeats on the expression of an internally located luciferase reporter could not be determined because all clones screened were found to have either integrated at a telomere or lost their telomeric DNA. The explanation for this finding is a possible area for future work but is not currently being investigated. Transgene expression was found not to vary extensively between subclones in either group. This work, as outlined for months 1-12, was completed ahead of schedule (described in 8, Appendix A1).

In addition to the experiments outlined for task 1 in the first year, I was able to show that telomere elongation (through overexpression of hTERT) specifically enhances silencing in telomeric clones (8, Appendix A1). Trichostatin A, a histone deacetylase inhibitor, was found to relieve silencing although the effect was not specific to telomeric clones (8, Appendix A1). I have also developed a second set of vectors and clones in which the luciferase reporter has been replaced with a fluorescent protein (dsRed) in order to be able to examine transgene expression at the single-cell level. Analysis of these clones in still in progress. I have studied extensively the expression patterns of several known subtelomeric genes in human fibroblasts by RT-PCR and Northern blotting (Appendix A2). Thus far I have found no evidence that these genes are regulated by telomere length, but plan to pursue new candidates as information becomes available (through the human genome project). We also have begun to take advantage of RNA interference (RNAi) technology to reduce the expression of various telomere binding factors as a first step toward identifying mediators of telomere position effect in human cells (Appendix A3). Confirmation of at least two positive results is underway.

Key Research Accomplishments

Original Statement Task 1. Establish the existence of telomere position effect in a human cancer cell line through a comparison of luciferase reporter genes inserted either at a telomere or internally, months 1-12:

- Inducible luciferase reporter plasmids were generated to insert either at the telomere or internally.
- Both internal and telomeric clones expressing the luciferase transgene were isolated.
- The position of the transgene in each clone was verified by Southern blot.
- Luciferase expression was compared between telomeric and internal clones and found to be reduced by 10-fold in the telomeric clones on average.
- The rates of induction and repression of luciferase expression for telomeric and internal clones were compared and found to be identical.
- Variation between subclones was found to be minor in both internal and telomeric clones.

Extension of Statement Task 1:

- Silencing of luciferase in telomeric clones was found to be enhanced 2 to 10-fold after telomere extension.
- The histone deacetylase inhibitor trichostatin A was found to relieve silencing of both telomeric and internal clones.
- A second set of telomeric and internal clones containing a fluorescent reporter has been developed and is being characterized.
- Several candidate endogenous telomeric genes were found not to be regulated by telomere length.

Reportable Outcomes

Publications

Baur, J.A., Zou, Y, Shay, J.W., and W.E. Wright. Telomere Position Effect in Human Cells. *Science* 292:2075-2077, 2001.

Abstracts

Baur, J.A., Zou, Y., Shay, J.W., Wright, W.E. Telomere position effect in human cells. Presented at the UT Southwestern GSO Poster Session. November, 2001.

Baur, J.A., Zou, Y., Shay, J.W., Wright, W.E. Telomere position effect in human cells. Presented at the UT Southwestern Integrative Biology Poster Session. April, 2002. (This was a new updated poster with the same title.)

Cell Lines Developed

HeLa cell lines containing either an internal or a telomeric luciferase reporter gene have been developed and characterized. Telomeres in several lines were extended through the introduction of telomerase to generate matched pairs having either short or long telomeres next to the reporter gene.

Conclusions

My work during this first year, including all the experiments originally outlined for "task 1", has resulted in the publication of the first proof that telomere position effect can occur in human cells. In addition, we have developed reagents that will allow us to compare human telomere position effect to the well-known yeast phenomenon, and to search for the protein mediators and the affected genes in human cells. The regulation of subtelomeric genes by telomere length could have extremely important implications for breast cancer, particularly as related to the subtelomeric breast cancer-related regulator of TP53 (BCPR) locus. Altered expression of subtelomeric genes could indicate the presence of cells with extremely short telomeres that could be expected to be at an increased risk of genomic rearrangements. Increased expression of subtelomeric gene expression may also have a more active role in the progression of breast cancer.

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inates contamination by microbes, which is an important criterion for maintaining food industry standards. Fermentative growth of naturally heterotrophic microalgae has resulted in dry biomass accumulation to 100 g/liter (*3*, *27*), which is 10 to 50 times the yields obtained by using light-dependent culture systems. Fermentation-based systems can reduce production costs of microalgae by an order of magnitude relative to that incurred by photosynthesis-based production; cost reduction analyses factor in expenses for both fixed-carbon supplementation and equipment operation (*28*). Commercial benefits of fermentation-based systems result from increased biomass, productivity, harvesting efficiency, and reduced losses from contamination. The ability to grow microalgae heterotrophically increases the feasibility for developing a large range of new algal products.

Marine ecosystems also depend on diatoms, which contribute substantially to the reduction of inorganic carbon in marine habitats. Such a contribution may increase substantially as the ecology of oceanic environments is altered (*29*–*32*). The exploitation of diatoms that can be genetically manipulated and that can grow heterotrophically will facilitate the use of mutants to augment our understanding of both photosynthesis and other metabolic pathways that are essential for competing in marine ecosystems.

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15. After 4 weeks in the dark, the transformants that grew were restreaked and maintained on 1.0% glucose. Liquid cultures were grown with 1.0% glucose at 20°C on an orbital shaker. All characterized transformants were generated from independent particle bombardments. Cells were grown at 20°C with continuous illumination at 75 μmol photons m^{-2} s^{-1} in Provasoli's enriched seawater medium with 10× the nitrogen and phosphorus by using Instant Ocean artificial seawater, at 0.5× concentration. Glucose was maintained between 5 and 10 g/liter. Growth rates were determined in 250-ml flasks (50 ml of media) with silicon foam closures. Daily samples measured cell numbers and nutrients. Flasks were stirred at 100 rpm. Fermentations were done in a 2-liter Applikont vessel by using an agitation rate of 100 rpm, dissolved oxygen was maintained at >20% saturation.
16. Cells in logarithmic phase growth were harvested, washed two times, and resuspended in fresh medium. Assays were initiated by adding unlabeled glucose and [D-^{14}C]glucose to 0.05 μCi/ml; the cells were maintained in the light. Samples were removed at 0, 2, 5, 10, and 15 min after the addition of labeled glucose. The cells were collected by filtration, washed with medium containing 1.0% unlabeled glucose, and transferred to scintillation vials.
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18. The cells were broken by using a MinibeadBeater by two cycles at full speed on ice. Cell membranes were pelleted by centrifugation at 100,000*g* for 30 min, solubilized in 2.0% SDS, resolved on 7.5% polyacrylamide gels, and transferred to nitrocellulose membranes.
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33. We thank D. Kyle and T. Allnutt for encouragement in the early parts of this work. Supported by NSF DMA-9560125 and funds from the Carnegie Institution of Washington.

20 February 2001; accepted 3 May 2001

Telomere Position Effect in Human Cells

Joseph A. Baur, Ying Zou, Jerry W. Shay,* Woodring E. Wright*

In yeast, telomere position effect (TPE) results in the reversible silencing of genes near telomeres. Here we demonstrate the presence of TPE in human cells. HeLa clones containing a luciferase reporter adjacent to a newly formed telomere express 10 times less luciferase than do control clones generated by random integration. Luciferase expression is restored by trichostatin A, a histone deacetylase inhibitor. Overexpression of a human telomerase reverse transcriptase complementary DNA results in telomere elongation and an additional 2- to 10-fold decrease in expression in telomeric clones but not control clones. The dependence of TPE on telomere length provides a mechanism for the modification of gene expression throughout the replicative life-span of human cells.

Most normal human cells lack the enzyme telomerase, which maintains telomeres, and as a consequence, telomeres shorten with each division until the cells reach replicative senescence (the Hayflick limit). This growth arrest is mediated by p53 and has been suggested to be the result of a DNA damage response to telomeres that have become too short (*1*–*3*). No mechanism has been demonstrated in vertebrates that can account for differences between young and old (but not yet senescent) cells. In *Saccharomyces cerevisiae*, telomere position effect (TPE) can result in the reversible silencing of a gene near a telomere by a mechanism that depends both on telomere length and on the distance to the gene (*4*–*6*). Because telomeres in most human cells shorten with age, TPE would provide a mechanism to incrementally alter phenotype with increasing cellular age (*7*). However, previous efforts to identify TPE in mammalian cells have not been successful (*8*–*10*). We demonstrate here the presence of TPE in human cells and that the strength of the silencing effect is dependent on telomere length.

We seeded de novo telomere formation in (telomerase-positive) HeLa cells by introducing a linear plasmid containing a luciferase reporter adjacent to 1.6 kb of telomere repeats (Web fig. 1) (*11*). Integration of a repeat-containing plasmid can result in breakage of the chromosome, followed by extension of the plasmid telomeric sequences by telomerase and loss of the distal chromosome fragment (*12*). Clones with a telomeric reporter were identified by Southern blotting of purified telomeres (Fig. 1A and Web fig. 2) (*11*) and confirmed by in situ hybridization (Fig. 1B). The mean length of the healed telomeres (after subtracting 3 kb of attached plasmid sequence) was estimated from Southern blots to be between 1.5 and 2 kb. Control clones were generated by transfection of an other-

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wise identical linearized construct that lacked telomere repeats. As expected with plasmid transfections, there was a high degree of variation within each group. The clones with a telomeric reporter nonetheless expressed luciferase at a 10 times lower average level than did the clones with an internal integration site (Fig. 2A).

We next sought to demonstrate that the lower expression levels in telomeric clones were the result of heterochromatin formation rather than of damage to the transgene or the presence of a mixed population of clones. Heterochromatin in mammalian cells is normally dependent on histone deacetylation. We therefore investigated whether treatment with trichostatin A (TSA), a highly specific inhibitor of histone deacetylases (*13*), could eliminate the telomeric silencing effect we had observed. Telomeric and internal clones were treated with TSA. After treatment, both sets of clones expressed the reporter at an enhanced level, representing a 2.6 ± 0.4–fold increase for the internal clones and a 51 ± 37–fold increase for the telomeric clones (Fig. 2B). The initial difference in the level of luciferase expression is thus histone deacetylase–dependent. Enhancement of transgene expression by histone deacetylase inhibitors has been noted previously (*14*). Luciferase expression returned to pre-experiment levels within 72 hours after withdrawal of the TSA (*15*). Although the TSA dose used in these experiments is somewhat cytotoxic, the toxicity did not play a role in increasing luciferase expression, because nonspecific treatment with toxic doses of hygromycin led to a moderate decrease in luciferase activity (*15*).

We next extended telomeres in order to establish the length dependence of the observed silencing effect. Increasing the telomerase activity of HeLa cells by infection with a human telomerase reverse transcriptase (hTERT)–encoding retrovirus causes them to elongate their telomeres (Fig. 3A), as has been observed in several other cell lines (*16*). We observed an additional 2- to 10-fold decrease in luciferase activity after telomeric clones were infected with a telomerase-containing retrovirus, as compared to control, vector-only infections (Fig. 3B). This change was not observed in clones with an internal luciferase reporter. These results demonstrate that this effect shares some similarities with yeast TPE and provides a mechanism by which the expression of subtelomeric human genes could increase with replicative age.

The strongest evidence against the existence of mammalian TPE comes from a comparison of mRNA levels for a telomeric *neo* gene in subclones of SV40-transformed human fibroblasts with varying telomere lengths (*8*). This cell line uses the ALT (alternative lengthening of telomeres) pathway to maintain its telomeres, a phenotype that involves altered telomere biology and a substantial increase in total

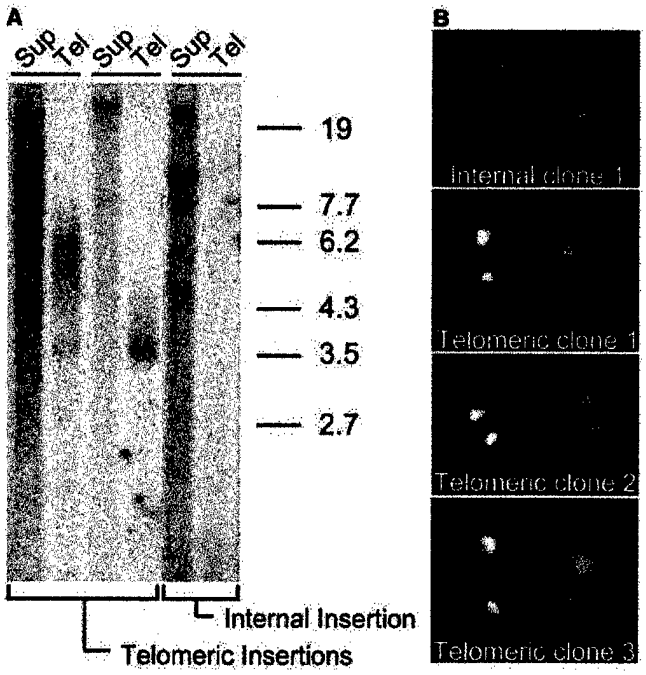

Fig. 1. Identification of telomeric clones by Southern blotting and in situ hybridization. (**A**) Genomic DNA was digested with Stu I, leaving the luciferase gene attached to the plasmid telomere sequences. Telomeres were then separated from bulk genomic DNA as described previously (*23*). Both the telomere fraction and the supernatant were separated on a 0.7% agarose gel, transferred to a Zeta-Probe blotting membrane (Bio-Rad, Hercules, California), and probed with luciferase sequences. Telomeric luciferase genes appear as a smear in the telomere fraction because of the heterogeneous lengths of the attached telomeres, whereas internally integrated genes appear as a discrete band in the supernatant fraction. Multiple integrations were noted in several of the internal control clones; however, the average was less than two (*11*). Markers shown are from λ DNA digested with *Sty* I (in kilobases). (**B**) Cells were fixed and probed simultaneously with the luciferase plasmid labeled with Spectrum Orange (Vysis, Downers Grove, Illinois), shown in red, and a fluorescein isothiocyanate–labeled oligonucleotide N3′-P5′ phosphoramidate probe complementary to telomere sequences [$(CCCTAA)_3$], shown in green. 4′,6′-diamidino-2-phenylindole staining is shown in blue. The top panel shows a clone with an internal integration site; the lower panels demonstrate the colocalization of the telomere and luciferase signals in three independent telomeric clones.

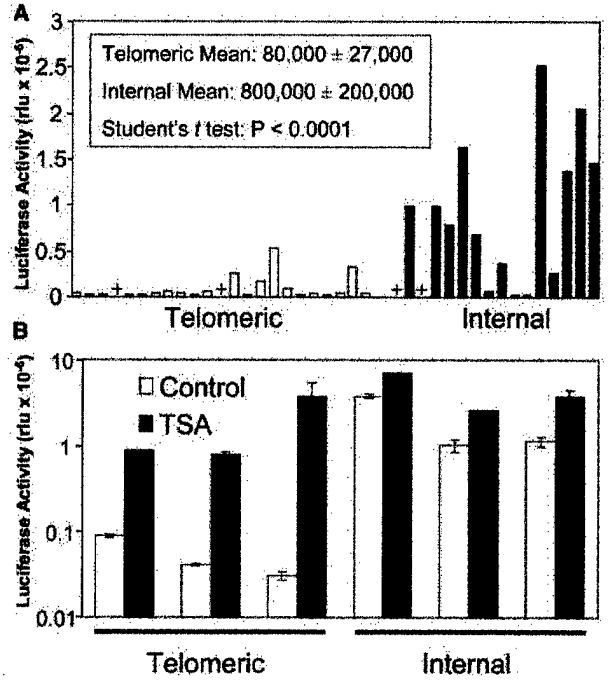

Fig. 2. Telomeric clones show a 10 times lower level of luciferase activity that is restored by a histone deacetylase inhibitor. (**A**) Puromycin-resistant clones were screened with a Luciferase Assay System (Promega, Madison, Wisconsin) on an Optocomp I luminometer (MGM Instruments, Hamden, Connecticut). The results for 23 telomeric and 15 internal integrations are shown. The plus signs indicate clones with a level of expression too low to be visible on this scale. (**B**) Silencing is relieved by the histone deacetylase inhibitor TSA. Three telomeric and three internal clones were treated with TSA (200 ng/ml) (Sigma, St. Louis, Missouri) for 24 hours. The medium was replaced, and the cells were incubated for an additional 24 hours before collection for luciferase assays. Note the switch to a logarithmic scale. rlu, relative light units.

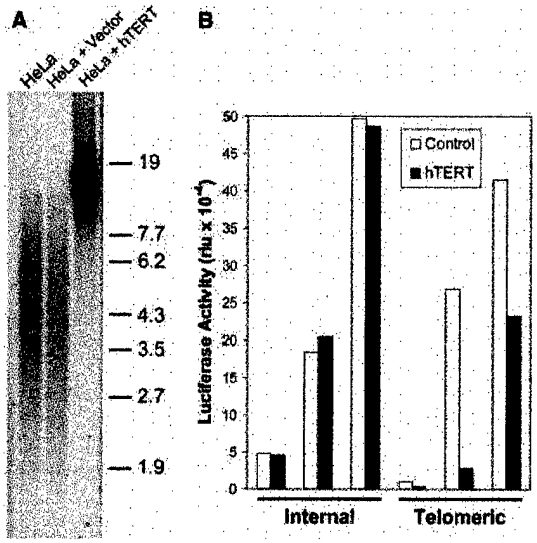

Fig. 3. Silencing in telomeric clones is enhanced by an increase in telomere length. (**A**) Infection of HeLa cells with an hTERT-encoding retrovirus causes telomere elongation, as demonstrated by terminal restriction fragment analysis. Mean telomere length increased from approximately 5 kb to almost 14 kb. Genomic DNA was digested with six restriction enzymes to degrade nonrepetitive sequences. Samples were then separated on a 0.7% agarose gel and probed with an oligonucleotide complementary to telomere repeats. Markers shown are λ *Sty* (in kilobases). (**B**) Telomeric clones infected with hTERT express 2 to 10 times lower levels of luciferase activity as compared to control, vector-only infections. Internal clones having comparable initial values retain full expression of the luciferase reporter after infection with hTERT.

telomeric DNA (*17*). It is possible that the extra telomeric sequences in ALT cells are titrating out factors essential for TPE, as has been observed in yeast (*18*), so that ALT cells might not exhibit TPE. Another report may have failed to identify TPE, because the healed telomere appears to have been extremely short and/or because it was located >50 kb from the nearest gene that could be examined (*9*). In at least one case, data consistent with a very mild mammalian TPE have been described (*19*), and the insertion of telomere repeats into an intron of the *APRT* gene of Chinese hamster cells was shown to cause a twofold reduction in the mRNA level (*20*).

A number of proteins have been reported to change in expression level as a function of the replicative age of the cell (*21*, *22*). The existence of TPE in mammalian cells raises the possibility that some presenescent changes could be "programmed" by the progressive shortening of telomeres with ongoing cell division, leading to altered patterns of gene expression that might affect both cell and organ function. It will be important to identify endogenous genes whose expression is influenced by telomere length in order to determine whether TPE actually influences the physiology of aging or cancer.

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Requirement of *CHROMOMETHYLASE3* for Maintenance of CpXpG Methylation

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Epigenetic silenced alleles of the *Arabidopsis SUPERMAN* locus (the *clark kent* alleles) are associated with dense hypermethylation at noncanonical cytosines (CpXpG and asymmetric sites, where X = A, T, C, or G). A genetic screen for suppressors of a hypermethylated *clark kent* mutant identified nine loss-of-function alleles of *CHROMOMETHYLASE3* (*CMT3*), a novel cytosine methyltransferase homolog. These *cmt3* mutants display a wild-type morphology but exhibit decreased CpXpG methylation of the *SUP* gene and of other sequences throughout the genome. They also show reactivated expression of endogenous retrotransposon sequences. These results show that a non-CpG DNA methyltransferase is responsible for maintaining epigenetic gene silencing.

Cytosine methylation plays a major role in determining the epigenetic expression state of eukaryotic genes. This methylation is most often found at the symmetrical dinucleotide CG (or CpG sites). CpG methylation is maintained by the well-studied DNMT1 subfamily of methyltransferases, which includes *Arabidopsis* MET1 (*1–3*). Methylation at sites other than CpG is also found in many organisms (*4*), but the mechanism by which this methylation is maintained is poorly understood. *Arabidopsis* can tolerate major disruptions in DNA methylation (*2, 3, 5*), making it useful for genetic analysis of methylation pattern-

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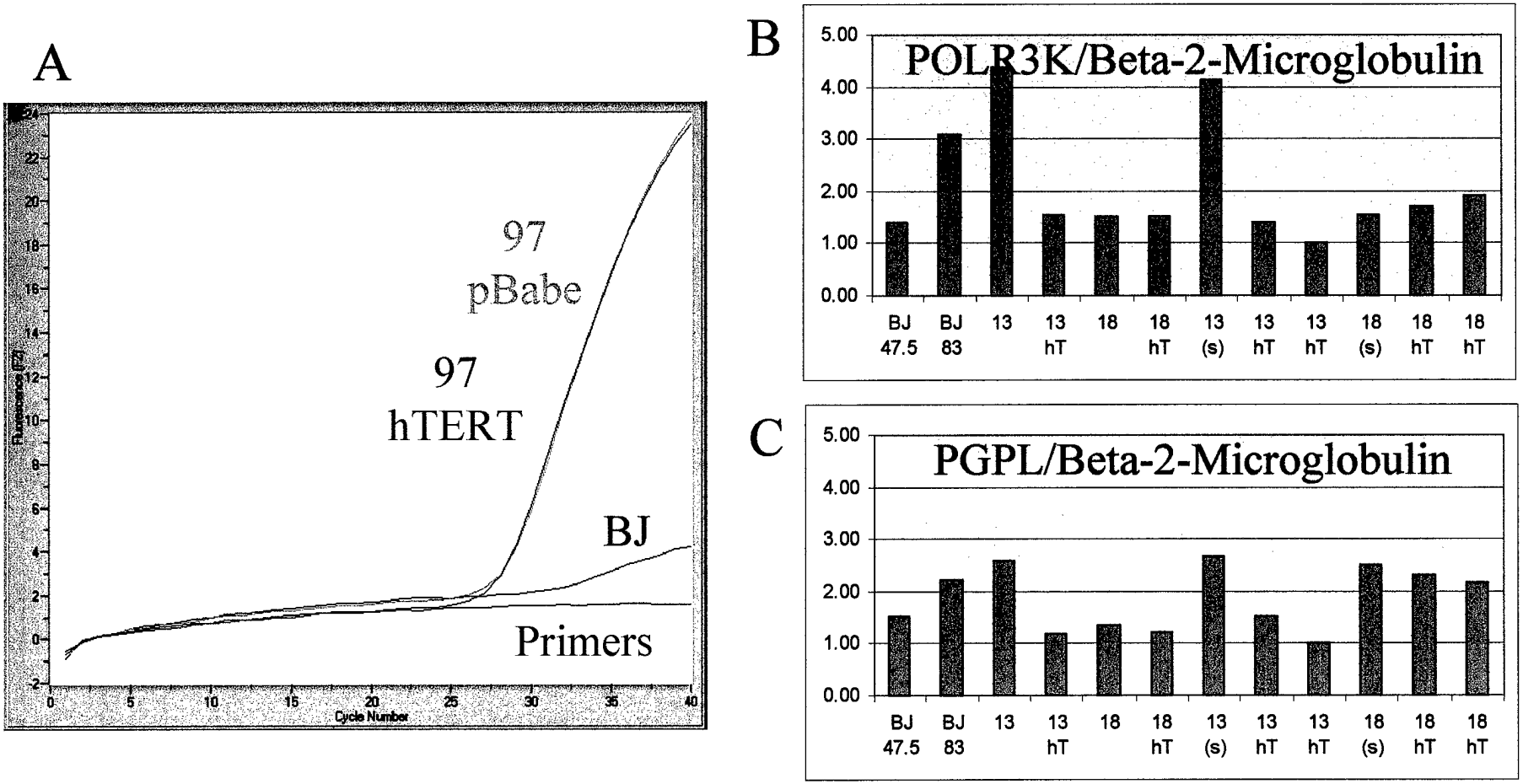
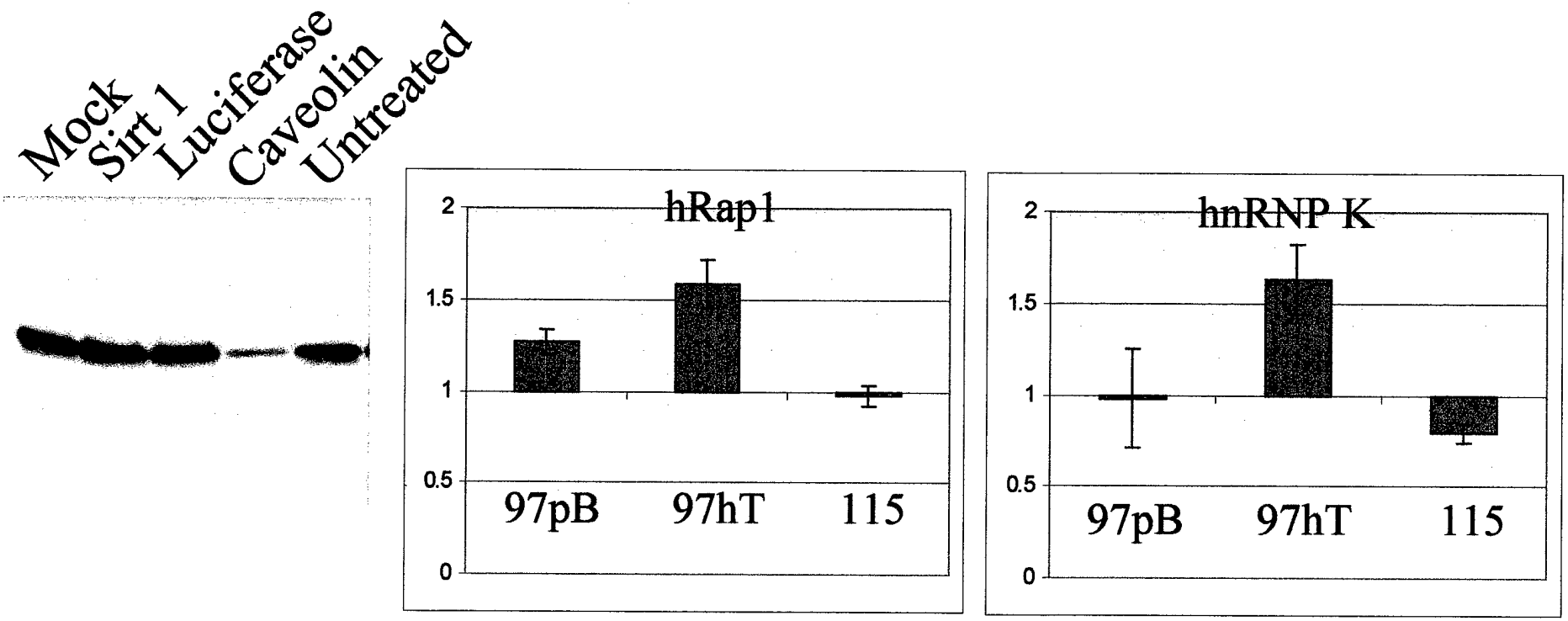


Appendix A2. TPE is not a property of all endogenous telomeric genes. (A) Real-time RT-PCR detection of endogenous telomerase mRNA in HeLa cells with short or long telomeres. Telomerase is itself a telomeric gene (on 5p) and endogenous message can be specifically amplified by targeting the 3'UTR, even in the presence of the exogenous telomerase used to lengthen telomeres. Telomerase was not found to be silenced in cells with long telomeres. (B) Expression levels (by Northern blot) for 2 telomeric genes, POLR3K and PGPL, normalized to beta-2-microglobulin. From left to right, the lanes represent decreasing telomere length until senescence (s), followed by extension of telomeres by telomerase (hT) for BJ fibroblasts. 13 is a BJ clone that reached a high number of population doublings with a very short telomere length before senescing. Using this method of normalization, both genes appear to exhibit TPE. (C) The same data presented in B is shown normalized instead to the average of beta-actin and GAPDH levels. By this normalization method, it appears that neither gene shows TPE and that beta-2-microglobulin is not an appropriate control gene for studying TPE.



Appendix A3. hRap1 and hnRNP K may be mediators of telomeric silencing. (A) Specific targeting of caveolin by RNA interference in HeLa cells. HeLa extracts from cells transfected with siRNAs targeting the indicated proteins were run on a Western blot and probed with anti-caveolin antibody. (B) Fold increase in the expression of a luciferase reporter next to a short telomere (97pB), a long telomere (97hT), or internal (115) after transfection with siRNA targeting hRap1. (C) Fold increase in luciferase expression after transfection of the same clones with siRNA targeting hnRNP K. Each point represents the average of 2-8 independent experiments normalized to mock transfection, cells transfected with an irrelevant target, or both.